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The incidence of breast cancer is increasing dramatically in the United States. Man-made environmental agents such as pesticides, polychlorinated biphenyls (PCBs), phthalate esters, and dioxin have been implicated in this increase. Many xenobiotics such as DDT and PCBs have weak estrogenic activity and may enhance breast cancer formation by an estrogenic effect on breast epithelial cell growth. These agents also inhibit gap junctional intercellular communication (GJIC). This effect may be involved in the ability of these agents to enhance growth and breast cancer formation. The studies outlined in this proposal will determine whether there is a link between xenobiotic inhibition of human mammary epithelial cell GJIC, growth, and estrogenicity. These studies are highly relevant to the prevention of breast cancer. An understanding of the relationship between xenobiotic inhibition of GJIC, estrogenic activity, and the enhancement of growth in human breast epithelial cells will lead to more widely acceptable, mechanism-based arguments that xenobiotics are involved in the etiology of human breast cancer. This should lead to more focused regulatory efforts to reduce exposure to these agents.

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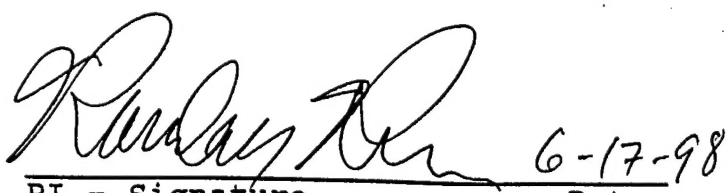

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BACKGROUND:

The incidence of breast cancer is increasing dramatically in the United States. Man-made environmental agents such as pesticides, polychlorinated biphenyls (PCBs), phthalate esters, and dioxin have been implicated in this increase (1-5). Several studies have identified increased levels of xenobiotics such as *p,p'*-DDE, the major human metabolite of DDT, in women with breast cancer (2-5). Many such xenobiotics have weak estrogenic activity and have been suggested to enhance breast cancer formation by an estrogenic effect on breast epithelial cell growth (6,7). However, the role of such xenoestrogens in human breast carcinogenesis is highly controversial. Several studies indicate no association between tissue levels of xenobiotics and breast cancer (8,9) and not all of the xenobiotics associated with breast cancer are estrogenic (9). Therefore, if synthetic chemicals are to be accepted as etiologic factors in breast cancer, relevant mechanisms must be demonstrated. Few mechanistic hypotheses have been tested, however.

Many of the xenobiotics that have been linked to human breast cancer inhibit gap junctional intercellular communication (GJIC) in nonbreast cells *in vivo* and *in vitro* (10,11). Substantial recent evidence indicates that a reduction of GJIC contributes to cell proliferation in xenobiotic-treated tissues and to the loss of growth control in neoplastic cells (12,13). Therefore, one potential mechanism for how xenobiotics might enhance breast cancer formation is that they inhibit breast epithelial cell GJIC resulting in enhanced growth. The studies outlined below will test this hypothesis.

Gap junctions are plasma membrane channels that connect the interiors of neighboring cells (12,13). The channels are approximately 1.5-2 nM in diameter and comprised of proteins known as connexins. At least thirteen connexins have been identified in mammals (13). GJ channels permit small (<1,000 Da) ions, nutrients, second messengers, metabolites, and other molecules to diffuse between neighboring cells, but prevent larger molecules and macromolecules from passing. The functions of this gap junctional intercellular communication (GJIC) include homeostasis, electrical synapsing (in certain neurons and muscle cells), and the regulation of cell growth (13). This latter function may be mediated by the intercellular exchange of signals such as cAMP that inhibit cellular growth, although these have not been identified (13).

Evidence that GJIC plays a role in growth regulation and neoplasia has been acumulating over the past three decades. Many neoplastically transformed cells have fewer gap junctions, reduced connexin expression, and decreased capacity for GJIC compared to nontransformed cells (14). A variety of growth enhancers, tumor promoters, and oncogenic agents inhibit GJIC whereas many growth inhibitors and differentiating agents enhance it (13). Stable transfection of gap junction-deficient neoplastic cells with connexin cDNAs has resulted in reduced growth *in vitro* and tumorigenicity *in vivo* of the transfected cells (15-19). Reduction of nontransformed cell GJIC by treatment with antisense connexin DNA enhanced cell growth *in vitro* (18) or blocked the cell's ability to suppress the growth of cocultured, transformed cells (21,22). These studies suggest that connexins are tumor suppressor genes.

PURPOSE:

Many environmental chemicals such as pesticides, phthalate esters, polychlorinated biphenyls, and dioxins have been demonstrated to be nongenotoxic carcinogens (tumor promoters) in two-stage rodent carcinogenesis bioassays. The model tissues have usually been the skin or liver. In some cases, xenobiotics may enhance tumor formation by increasing

initiated cell growth relative to surrounding noninitiated cells (25). Many of these agents also inhibit gap junction function and/or connexin expression *in vivo* and *in vitro* (10,11), demonstrating a link between the upregulation of growth and the downregulation of GJIC.

Several of the xenobiotics that have been implicated as etiologic factors in human breast cancer are also weakly estrogenic (6,9). No studies, however, have examined whether xenobiotics affect GJIC in human mammary epithelial cells and the relationship to growth and estrogenic activity. The inhibition of GJIC might lead to increased cell growth and the enhancement of neoplastic transformation in the breast. **The purpose of this project is to determine whether xenobiotics block GJIC and/or enhance growth in human mammary epithelial cells and how these effects correlate with estrogen receptor (ER) status, neoplastic transformation, and xenobiotic estrogenicity. The hypothesis is that pesticides that block GJIC will stimulate cell growth irrespective of xenoestrogenicity, estrogen receptor status, and neoplastic transformation.**

SCOPE OF THE RESEARCH:

We are testing several DDT-related compounds (*p,p'*-DDT, *p,p'*-DDE, *p,p'*-DDD, and *o,p'*-DDT) for their effects on GJIC and growth of ER^+ and ER^- normal and neoplastic human mammary epithelial cells. These agents inhibited GJIC in rodent fibroblasts (26,27) and a recent study (28) showed that *p,p'*-DDT blocked GJIC in NHMEC, but did not examine growth effects. These latter cells were obtained from reduction mammoplasties. We are also using ß-estradiol, an estrogenic steroid, as a positive control. We are correlating changes in GJIC with cell growth, estrogenicity, estrogen-receptor status, and neoplastic transformation. We will also identify the biochemical and molecular mechanisms of action of these compounds on GJIC in HMEC. Specifically, we will determine whether connexin expression and localization are affected by these compounds (29). This will enable comparisons with other tissues and cell types and will facilitate the development of antagonists. Finally, by correlating xenobiotic effects on GJIC with changes in HMEC proliferation and estrogenic activity, we will provide mechanistic information on the role of altered GJIC in breast epithelial cell growth and neoplasia and how this is related to estrogenicity.

PREVIOUS STUDIES RELATED TO THIS RESEARCH:

Connexin43 (Cx43) and connexin26 (Cx26) are expressed in normal human mammary epithelial cells (NHMEC) (23,24). When gene expression in neoplastic breast epithelial cells was compared to nontransformed breast epithelial cells using the technique of subtractive hybridization, Cx26 was identified as a gene whose expression was reduced in the neoplastic cells (23). Subsequently, GJIC and connexin expression in several human breast carcinoma cell lines and primary breast carcinomas were analyzed and the results demonstrated that GJIC and the expression of both Cx26 and Cx43 were nearly undetectable in the neoplastic cell lines and tumors (24). Thus, the reduction of connexin expression and GJIC appears to contribute to the neoplastic phenotype of human breast carcinoma cells as in other neoplastic cells.

As mentioned above, DDT-related compounds have been shown to block GJIC in many types of cells including NHMEC (28). No studies, however, have correlated this with ER status of the cells or with effects on cellular growth.

Recent studies from our group (29-33) and others (34,35) are defining how xenobiotics alter GJIC *in vivo* and *in vitro*. Our data indicate that the inhibition of GJIC by *p,p'*-DDT in

WB-F344 rat liver epithelial cells which express Cx43 occurs as both rapid and prolonged effects (29,32). Rapid inhibition occurs within 15 min of exposure of WB cells to *p,p'*-DDT without changes in gap junction number, gap junction size, Cx43 content, or Cx43 phosphorylation and is most likely due to closure or blockage of gap junction channels. The rapid reduction in channel permeability may be due to changes in $[Ca^{+2}]_i$, $[H^+]_i$, free radicals, or plasma membrane fluidity. Inhibition after prolonged exposure (0.5-6 h) to *p,p'*-DDT involves decreases in Cx43 content and in the number of gap junctions. This is due to gap junction endocytosis and degradation in lysosomes. These changes also appear to be posttranscriptional since no appreciable changes in Cx43 mRNA levels were detected (29,32).

STATEMENT OFWORK:

The technical objectives of this project are:

1. Characterize the dose-responsive effects of DDT-related xenobiotics on GJIC in human mammary epithelial cells.
2. Determine whether DDT-related xenobiotics affect the growth of human mammary epithelial cells.
3. Identify the biochemical and molecular mechanism(s) by which DDT-related xenobiotics alter GJIC in human mammary epithelial cells.

EXPERIMENTAL METHODS:

1. Culture of human mammary epithelial cells: Proliferating cultures of NHMEC (samples 4144 and 4678) were obtained from Clonetics, Corp. (San Diego, CA) and cultured in serum-free/phenol red-free growth medium also obtained from Clonetics. These cells were derived by Clonetics from reduction mammoplasties and consist of cells from the mammary gland terminal ducts, the most common site of breast carcinoma development (36). NHMEC proliferate for at least 15 population doublings *in vitro* and are cultured by routine methods.

Several immortalized human mammary epithelial cell lines have been obtained from Dr. Bonnie Sloane (Wayne State University) and are also being used. These include nontransformed MCF-10A, H-ras-transfected MCF-10AneoT, neoplastic MCF-7, and neoplastic BT-20 cells. All cell lines were cultured in phenol red-free Dulbecco's MEM/F12 medium supplemented with 5% fetal bovine serum (FBS) and gentamicin (40 μg/ml). MCF-10A and MCF-10AneoT were also cultured in the same medium with additional growth factor supplements (20 ng/ml EGF, 10 ng/ml insulin, 100 ng/ml cholera toxin, and 500 ng/ml hydrocortisone). During treatments with xenobiotics, charcoal dextran-stripped FBS was used in place of the normal FBS.

2. Determination of xenobiotic toxicity in mammary epithelial cells: The toxic effects of the DDT-related compounds was determined in these cells by trypan blue dye staining. Test agents were first dissolved in dimethylsulfoxide (DMSO) then applied to the cells (1 μl/ml culture medium). Control cultures were treated with DMSO (1 μl/ml). The cultures were sampled 1, 3, and 7 d after treatment and stained with 0.4% trypan blue. Viable and nonviable (blue) cells were identified and counted microscopically as we have reported (30).

3. Xenobiotic effects on mammary epithelial cell growth: Xenobiotic effects on mammary cell growth *in vitro* was determined as we have reported (37). Cells were plated into 24 well multi-well dishes (25,000 cells/well), treated with xenobiotics, and the number of cells

per well was determined on day 7 of treatment by trypsinizing the cells and counting them with a hemacytometer. Triplicate wells were sampled per xenobiotic dose.

4. Dye microinjection assay for GJIC: GJIC in these cells was assayed by microinjection of fluorescent Lucifer Yellow (LY) dye as we have described (29). The cells were cultured in 35 mm dishes, treated with the test agents for 1-7 d, then microinjected. Cells were impaled with LY-filled glass micropipets and dye was loaded into the cells by iontophoresis. Cells were observed under the fluorescent microscope for evidence of dye transfer to neighboring cells. GJIC was quantified as the percentage of neighboring cells adjacent to microinjected cells that took up dye. Ten cells per dish were injected for each treatment dose and duration and triplicate dishes were run per treatment group.

5. Immunostaining of Cx43 and Cx26 gap junctions: We have obtained highly specific mouse monoclonal and rabbit polyclonal antibodies to both Cx43 and Cx26 from Zymed Corp. (South San Francisco, CA). These will be used to stain Cx43 and Cx26 gap junctions in xenobiotic-treated mammary epithelial cells by indirect immunofluorescence as we have reported (29). After staining, gap junction spots will be counted morphometrically (29) and changes in the number of gap junction spots per cell will be correlated with reductions in dye-coupling. As noted with rat liver epithelial cells (29-33), we expect to see dose-dependent reductions of Cx43 and Cx26 gap junction number in mammary epithelial cells and that these changes will only occur after prolonged (several hours) treatment.

6. Western blot assay of Cx43 and Cx26 protein: Cx43 and Cx26 protein levels in xenobiotic-treated mammary cells were analyzed by Western blotting as we have described (29) using the above antibodies to Cx43 and Cx26.

7. Northern blot assay of Cx43 and Cx26 mRNA: Steady-state levels of Cx43 and Cx26 mRNA in xenobiotic-treated mammary epithelial cells were analyzed by Northern blotting as we have described (29). The blots were hybridized with probes generated by random primer labeling of Cx43 and Cx26 full-length cDNAs available in my laboratory. The blots were stripped and rehybridized with a glyceraldehydephosphate dehydrogenase (GAPDH) probe to check RNA loading and transfer.

RESULTS AND DISCUSSION:

1. Culture of human mammary epithelial cells: We have successfully cultured NHMEC-4144, NHMEC-4678, and the immortalized mammary cell lines as described above. The NHMEC were passaged by trypsinization 3-4 times before they senesced; in the studies described below, we used the cells at passages 1 and 2.

2. Determination of xenobiotic toxicity in mammary epithelial cells: The toxic effects of *p,p'*-DDT, *p,p'*-DDE, *p,p'*-DDD, *o,p'*-DDT, and β-estradiol at concentrations of 0.001, 0.01, 0.1, and 1 μM were determined in the above cells by trypan blue dye staining. Control cultures were treated with DMSO (final concentration of 0.1%). None of the DDT-related compounds or the estradiol were toxic (i.e., they did not increase trypan blue staining) after 1 and 7 d of treatment (data not shown).

3. Xenobiotic effects on mammary epithelial cell growth: The effects of DDT-related compounds and β-estradiol on the growth of mammary cells were determined after culturing the cells for 7 d in the presence of each agent. The results are shown in Figures 1-8 and summarized in Table 1. In the ER^- NHMEC, MCF-10A (plus growth factors), MCF-10AneoT (plus growth

factors), and BT-20 cells, the compounds did not affect cell growth. Increases in cell growth were seen, however, when MCF-10A and MCF-10AneoT cells were cultured without additional growth factors and in the ER^+ MCF-7 cells.

The enhancement of cell growth in MCF-7 cells was expected and has been attributed to the estrogenic effects of the xenobiotics (34,35). However, the enhancement in ER^- MCF-10A and MCF-10AneoT cells only in the absence of growth factors was surprising. This result suggests that the pesticides can induce cell growth in mammary epithelial cells in an ER-independent manner. This is also supported by the fact that β-estradiol did not increase growth in these cells. Why the pesticides increased growth only in the absence of added growth factors is unclear, but suggests that the compounds may have substituted for the growth factors or used a similar pathway. This effect, however, appears to be specific for the MCF-10A and MCF-10AneoT cells since growth of the BT-20 cells was not enhanced by the pesticides even though the cells were also cultured in the absence of added growth factors. The NHMEC cells were also refractory to the pesticides, but their medium contains added growth factors whose composition is proprietary (Clonetics Corp.).

4. Dye microinjection assay for GJIC: The effects of DDT-related compounds and β-estradiol on GJIC in the mammary cells was determined after 1 and 7 d treatment and the results are shown in Figures 1-8 and Table 1. These agents either decreased or had no effect on GJIC (dye-coupling percentage) and there were no apparent correlations between compound estrogenicity, ER status, growth factor supplemenation, or neoplastic transformation. Interestingly, the nontransformed cells (NHMEC and MCF-10A cells) had higher levels of GJIC than the neoplastic cells. This was expected based upon previous studies (24).

5, 6, and 7. Immunostaining of Cx43 and Cx26 gap junctions; Western blot assay of Cx43 and Cx26 protein; and Northern blot assay of Cx43 and Cx26 mRNA: The effects of DDT-related compounds and β-estradiol on Cx43 content in the mammary cells was determined after 1 and 7 d treatment. These studies are in progress, but some results are shown in Figures 9 and summarized in Table 1. The data thus far suggest that in most cases, the compounds do not affect Cx43 expression. We did note an increase in Cx43 content in MCF-7 cells after treatment with β-estradiol. This result needs to be verified, however.

CONCLUSIONS:

The data generated to date indicate that the DDT-related pesticides enhanced cell growth in MCF-7 cells and MCF-10A and MCF-10AneoT cells, but the effect in the latter two lines occurred only when they were cultured in the absence of additional growth factors. The pesticides did not affect the growth of the other types of cells. These agents also blocked GJIC in a cell-specific manner and this blockage was not related to effects on cell growth.

Our data suggest that the enhancement of cell growth in human mammary epithelial cells by DDT-related agents may occur by ER-dependent and ER-independent mechanisms. Similarly, the inhibition of GJIC by these compounds does not appear to involve the ER and does not appear to be causally related to the enhancement of cell growth.

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Warner, K.A., Fernstrom, M.J., and Ruch, R.J. (1998) Growth enhancement of normal and neoplastic human breast epithelial cells by xenoestrogenic pesticides is unrelated to the inhibition of gap junctional intercellular communication. *Carcinogenesis*, in preparation.

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GRADUATE DEGREES RESULTING FROM THE AWARD

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Table 1. Summary of the effects of DDT-related compounds on growth, GJIC, and Cx43 expression in normal, immortalized, and neoplastic human mammary epithelial cells.

Cell type	ER status	Compound	Growth	GJIC - 1 d	GJIC - 7 d	Cx43 mRNA	Cx43 protein
NHMEC-4144	negative	p,p'-DDT	NE	D	NE	ND	ND
		p,p'-DDE	NE	NE	NE	ND	ND
		p,p'-DDD	NE	D	NE	ND	ND
		o,p'-DDD	NE	D	NE	ND	ND
		β-estradiol	NE	NE	NE	ND	ND
NHMEC-4678	negative	p,p'-DDT	NE	D	D	NE	NE
		p,p'-DDE	NE	NE	NE	NE	NE
		p,p'-DDD	NE	NE	D	NE	NE
		o,p'-DDD	NE	D	D	NE	NE
		β-estradiol	NE	NE	D	NE	NE
MCF-10A (minus GF)	negative	p,p'-DDT	I	D	D	ND	ND
		p,p'-DDE	NE	D	D	ND	ND
		p,p'-DDD	I	D	D	ND	ND
		o,p'-DDD	I	D	D	ND	ND
		β-estradiol	NE	NE	NE	ND	ND
MCF-10A (plus GF)	negative	p,p'-DDT	NE	D	D	ND	ND
		p,p'-DDE	NE	D	D	ND	ND
		p,p'-DDD	NE	D	NE	ND	ND
		o,p'-DDD	NE	D	NE	ND	ND
		β-estradiol	NE	D	D	ND	ND

Table 1, continued.

Cell type	ER status	Compound	Growth	GJIC - 1 d	GJIC - 7 d	Cx43 mRNA	Cx43 protein
MCF10AneoT (minus GF)	negative	p,p'-DDT	I	NE	NE	ND	ND
		p,p'-DDE	NE	NE	NE	ND	ND
		p,p'-DDD	I	NE	NE	ND	ND
		o,p'-DDD	I	NE	NE	ND	ND
		β-estradiol	NE	NE	NE	ND	ND
MCF10AneoT (plus GF)	negative	p,p'-DDT	NE	D	D	NE	D
		p,p'-DDE	NE	D	NE	NE	NE
		p,p'-DDD	NE	D	NE	NE	NE
		o,p'-DDD	NE	D	NE	NE	NE
		β-estradiol	NE	D	NE	NE	NE
MCF-7	positive	p,p'-DDT	I	D	D	NE	NE
		p,p'-DDE	I	D	D	NE	NE
		p,p'-DDD	I	NE	D	NE	NE
		o,p'-DDD	I	D	NE	NE	NE
		β-estradiol	I	NE	NE	I	I
BT-20	negative	p,p'-DDT	NE	NE	D	ND	ND
		p,p'-DDE	NE	NE	NE	ND	ND
		p,p'-DDD	NE	NE	NE	ND	ND
		o,p'-DDD	NE	NE	NE	ND	ND
		β-estradiol	NE	NE	NE	ND	ND

D, decreased.
I, increased.
ND, not determined.
NE, no effect.

NHMEC-4144 Cells

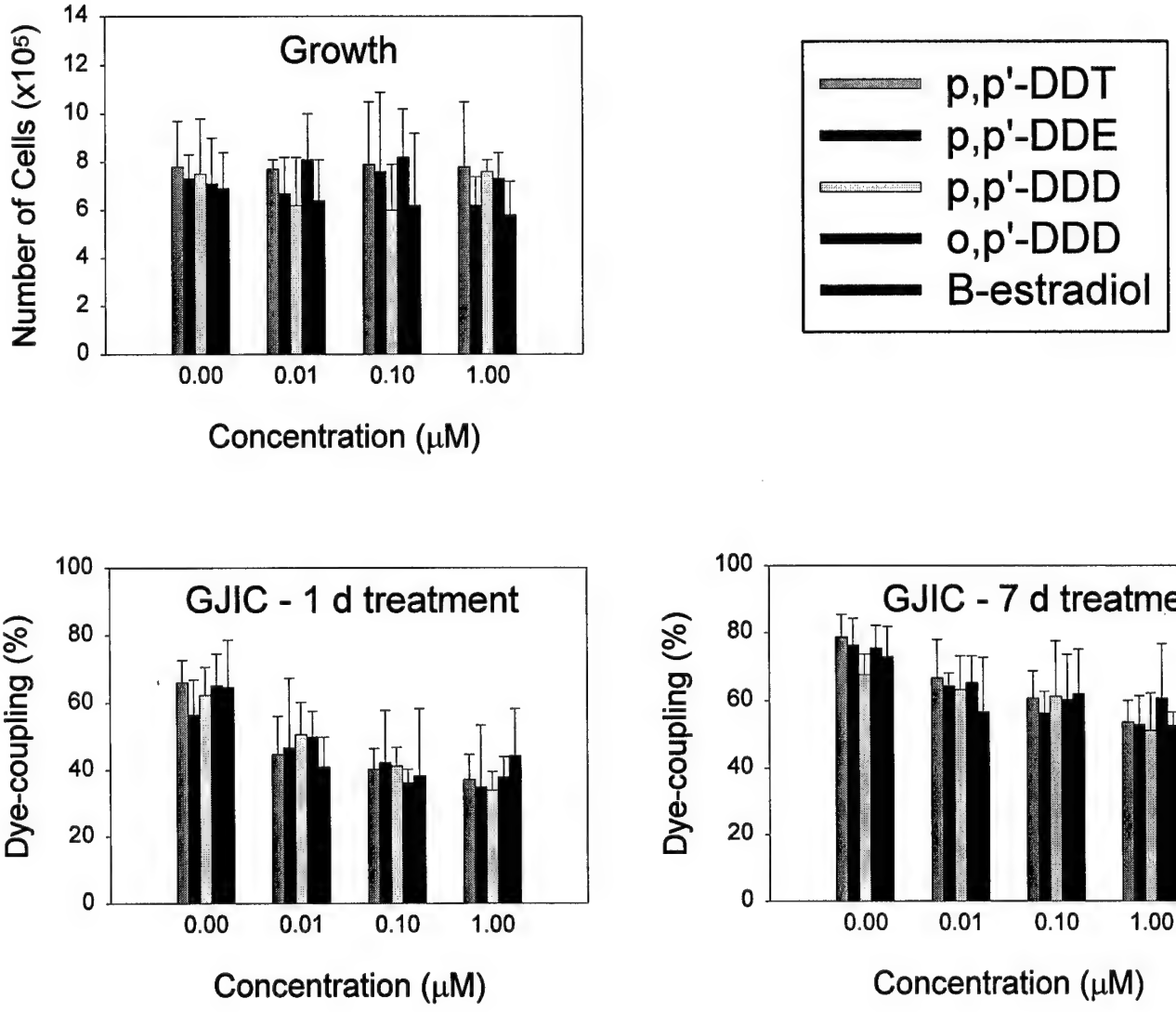


Figure 1

NHMEC-4678 Cells

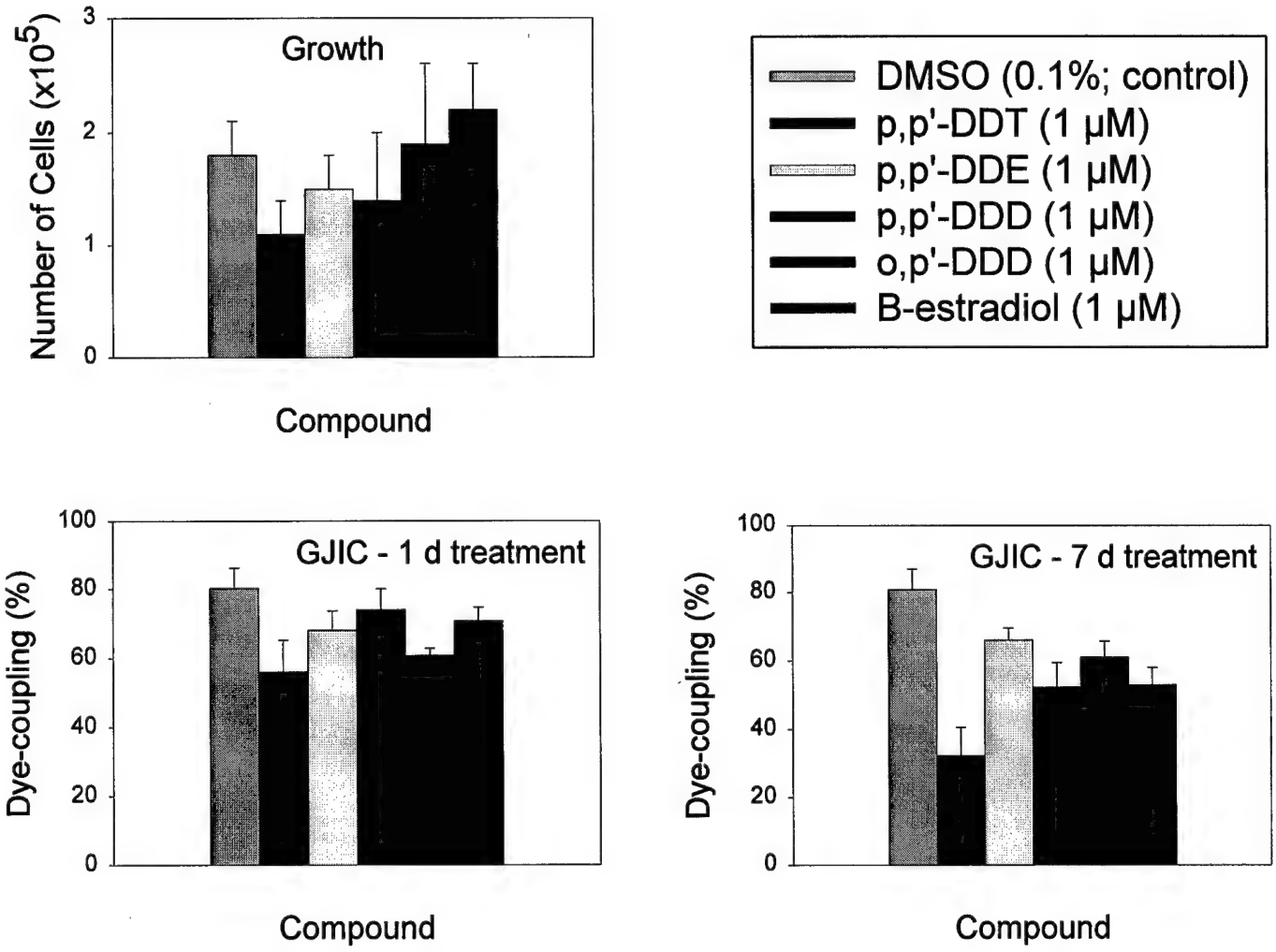


Figure 2

MCF-10A Cells (minus growth factors)

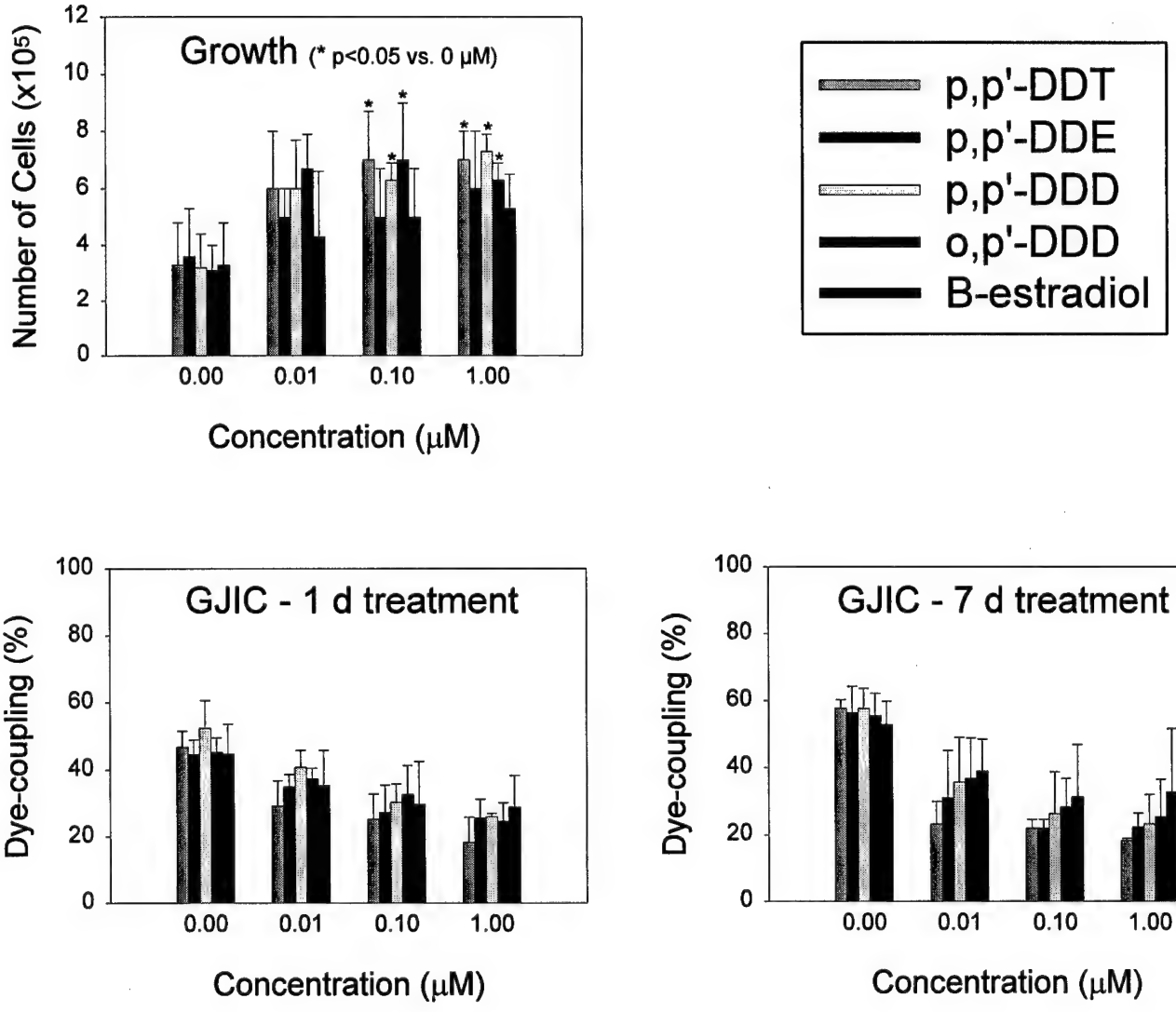
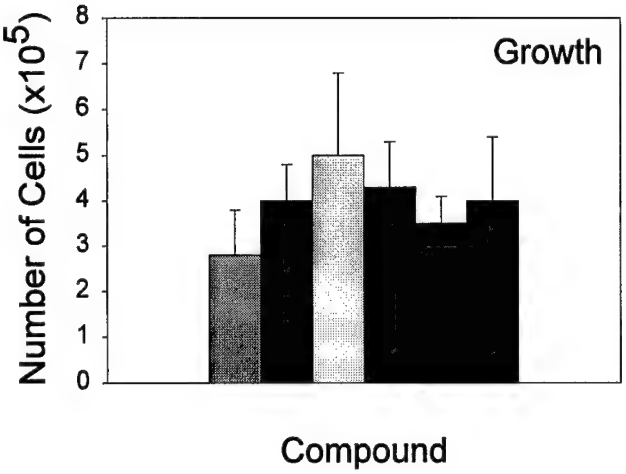
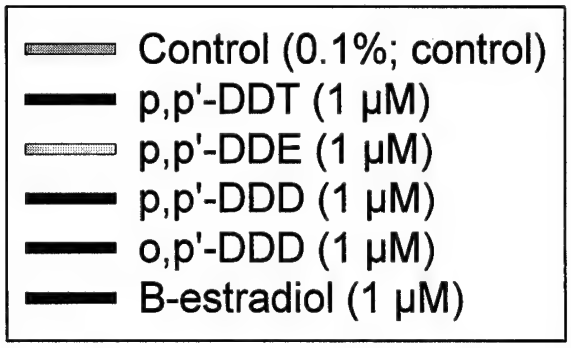
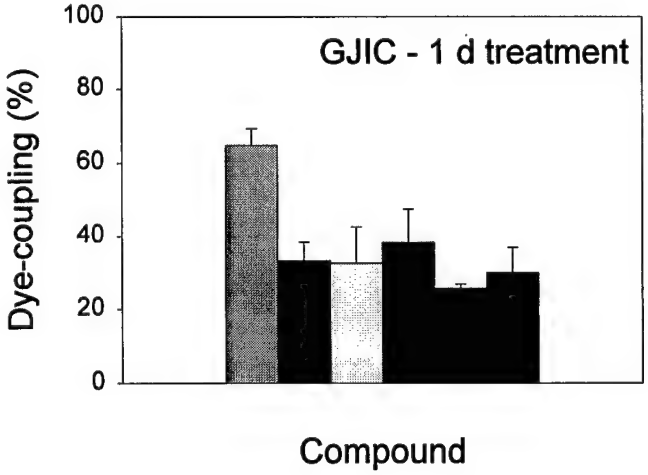


Figure 3

MCF-10A Cells
(plus growth factors)







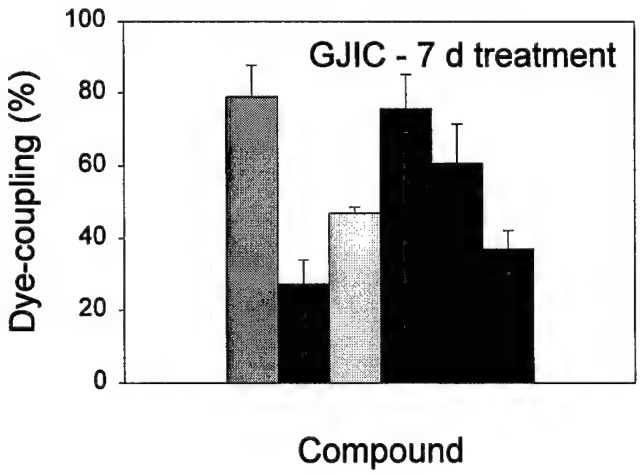
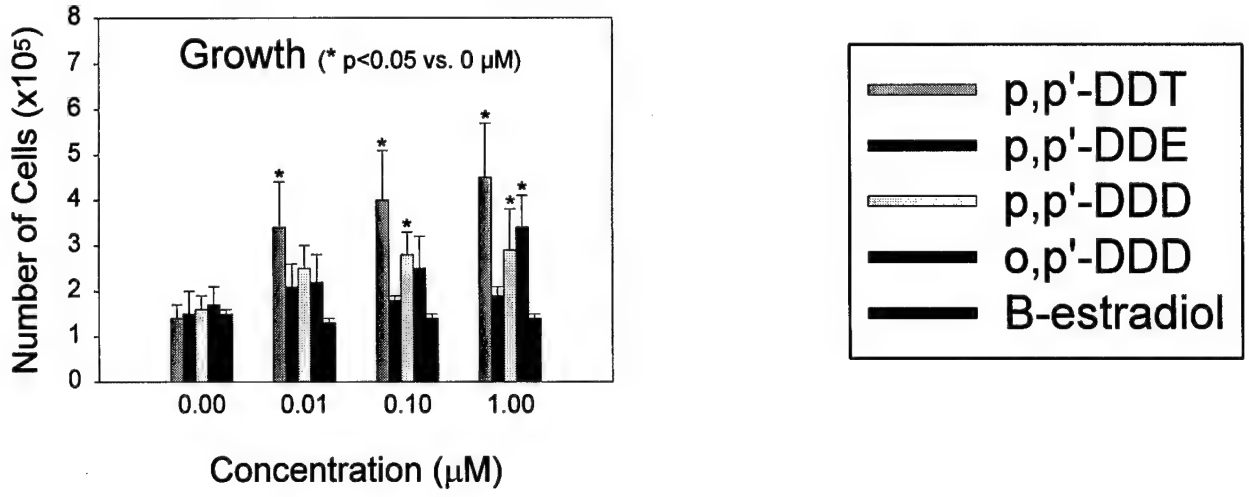
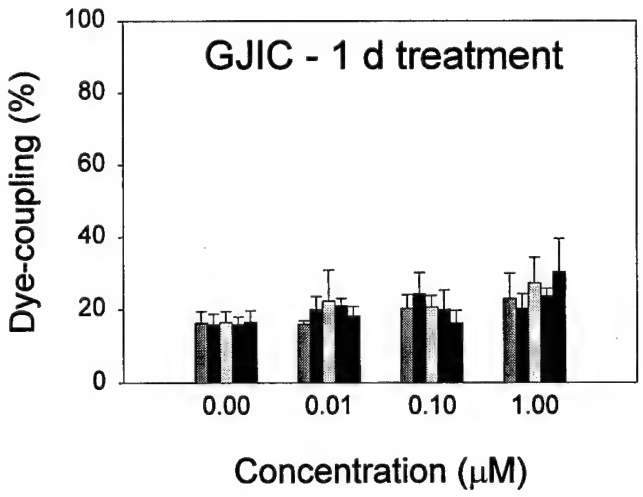


Figure 4

MCF-10AneoT Cells (minus growth factors)





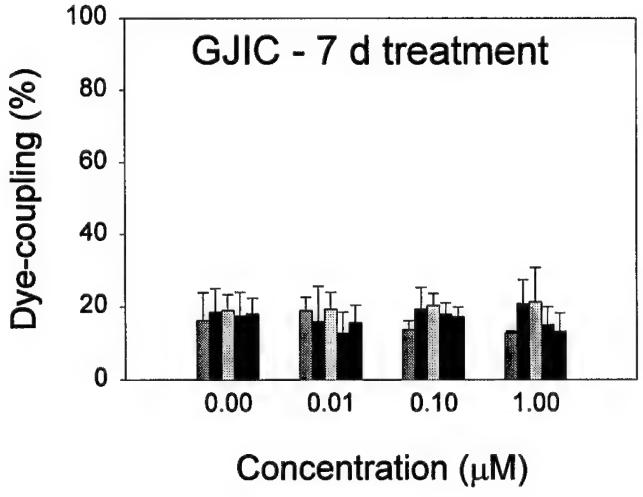


Figure 5

MCF-10AneoT Cells (plus growth factors)

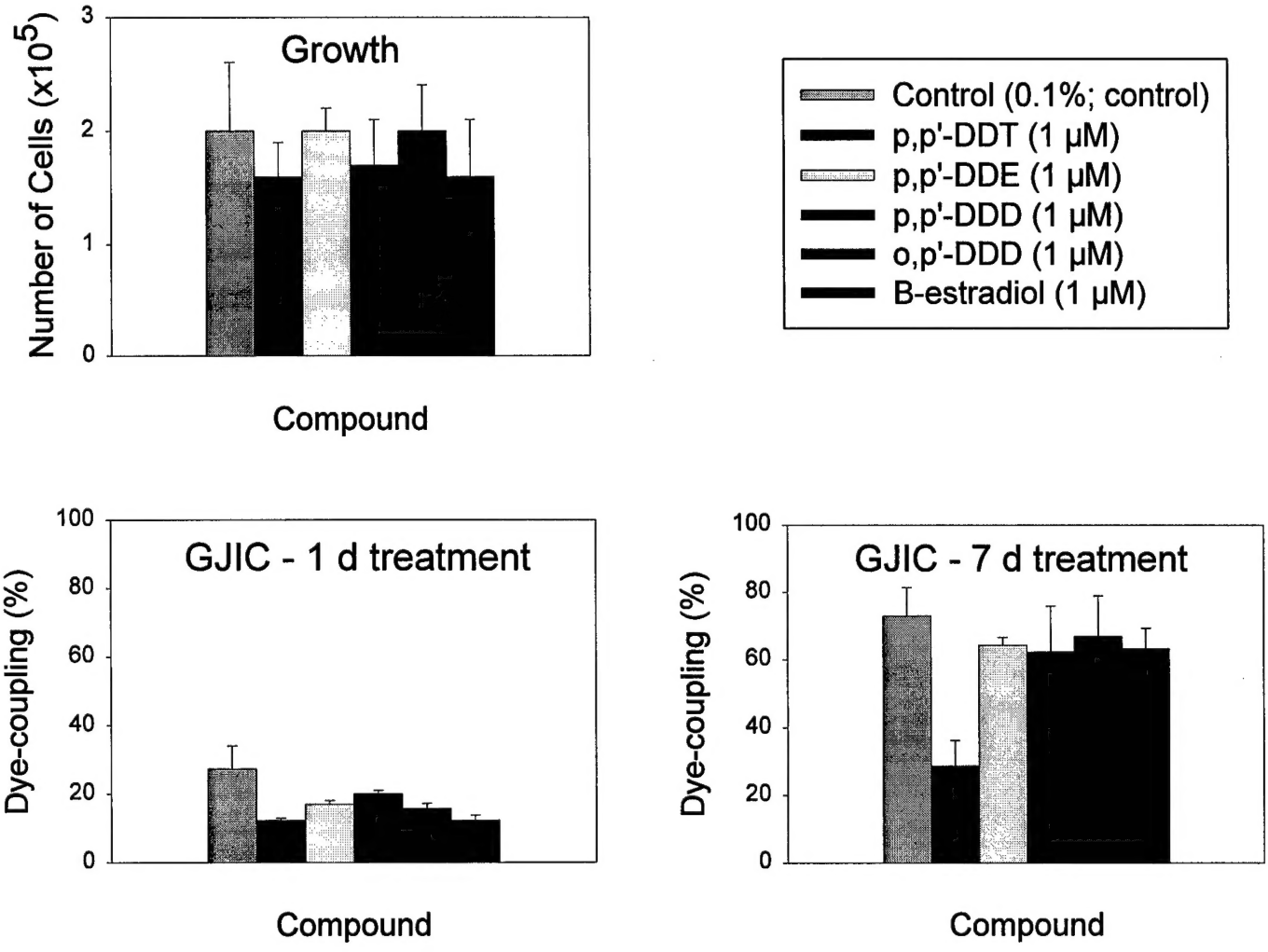


Figure 6

MCF-7 Cells

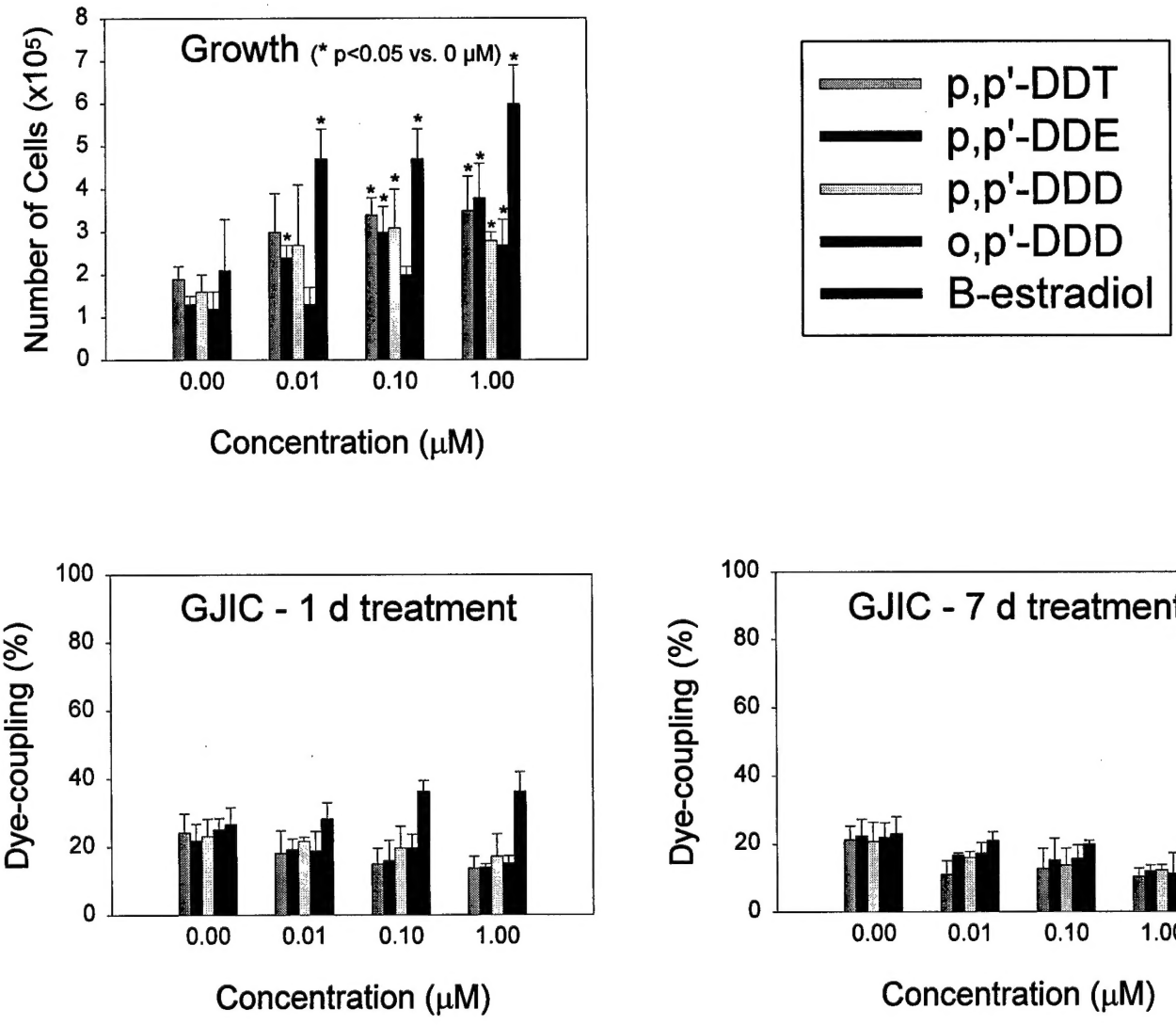
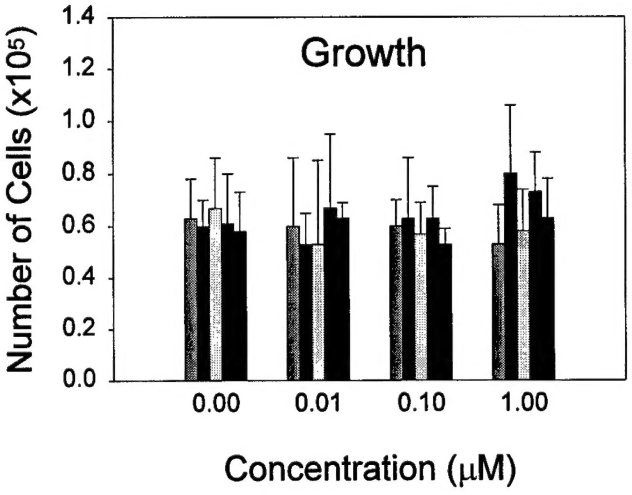
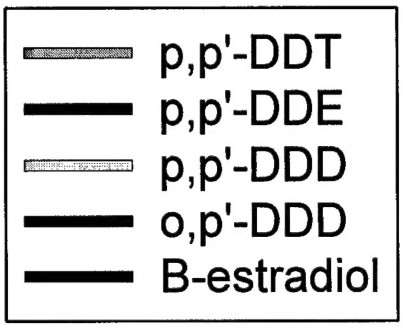
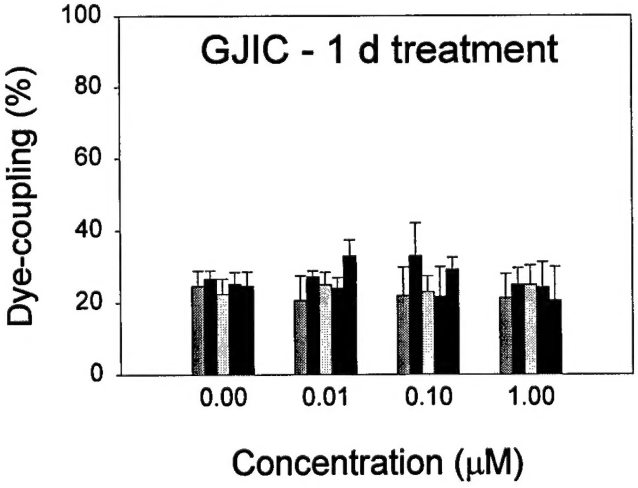


Figure 7

BT-20 Cells







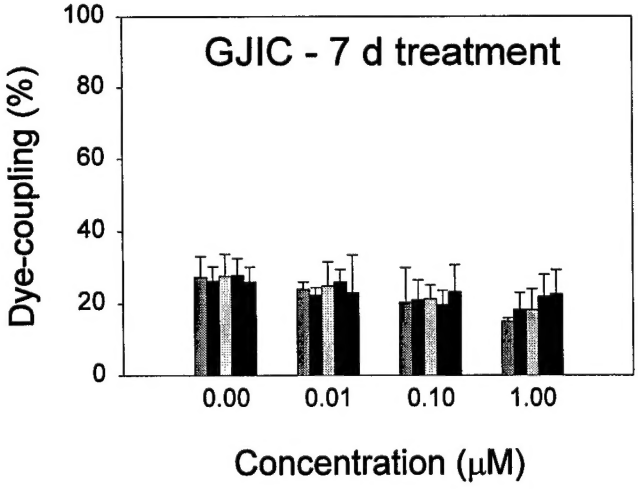


Figure 8

Cx43 Content in NHMEC-4678 Cells

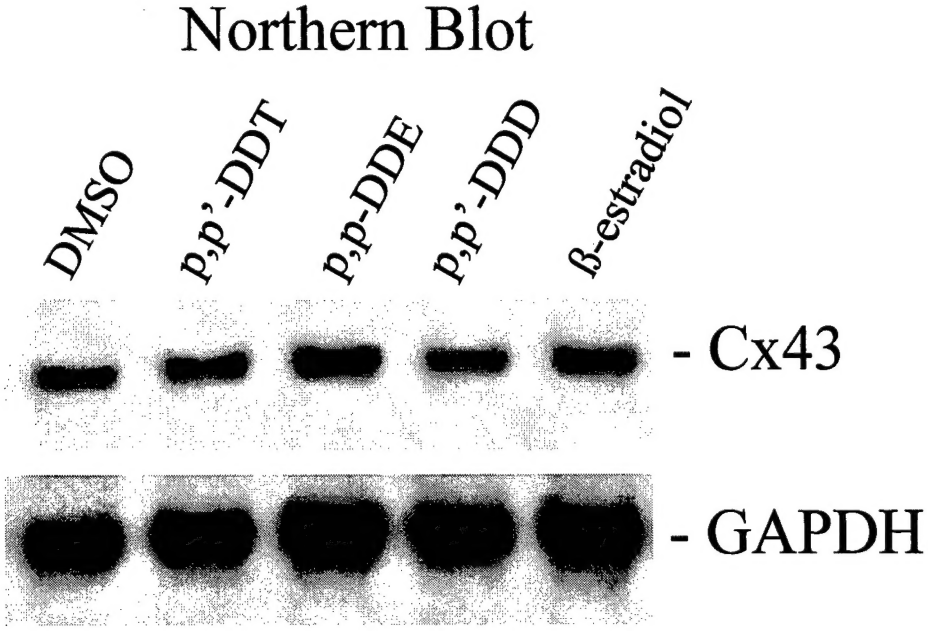


Figure 9